J. Jpn. Bot. **85**: 263–269 (2010)

Molecular Identification of Turmeric (*Curcuma longa*, *Zingiberaceae*) with High Curcumin Content

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(Received on April 2, 2010)

Curcumin is mainly found in *Curcuma longa* L. (*Zingiberaceae*) and is an important constituent of spices and medicines. Previous investigations of *C. longa* indicated that the amount of curcumin was different among various strains of this species. It is difficult to identify strains with a high content of curcumin within *C. longa*, so molecular identification may provide useful. To conduct an accurate and easy identification of the high content strain of curcumin, we evaluated chloroplast DNA (cpDNA) sequences of various individuals of *C. longa* by the method of PCR amplification. The result indicates that the only strains with a high content of curcumin have total of 20-bp insertion or deletion (indel) in the *atpF* intron of cpDNA and have a one site digesting on the indel by the restriction enzyme of *ScaI*. From these results, the PCR amplification of the *atpF* intron and the PCR-RFLP using the *ScaI* for *C. longa* allows the accurate identification of the strains with high curcumin content.

Key words: Chloroplast DNA, *Curcuma*, curcumin, molecular analysis, Turmeric.

Curcumin is the main curcuminoid found in the spice turmeric, *Curcuma longa* L. (*Zingiberaceae*). It has been used in the Indian and Chinese systems of medicines to treat wounds and sprains, and gastrointestinal, pulmonary, and liver disorders (Aggarwal et al. 2007). Many pharmacological studies have been conducted to describe multiple biological actions of curcumin. These studies have demonstrated that curcumin possesses antioxidant (Sreejayan and Rao 1994), anti-inflammatory (Ammon and Wahl 1991), anticarcinogenic (Johnson et al. 2009), antimicrobial (Limtrakul et al. 1997), hepatoprotective (Kiso et al. 1983), hypoglycemic (Babu and Srinivasan 1995), thrombosuppressive (Srivastava et al. 1985), and antiarthritic (Deodhar et al. 1980) activities. Moreover, curcumin has also demonstrated neuroprotective effects in animal models of Alzheimer´s disease (Ma et al. 2009) and Parkinson´s disease (Jagatha et al. 2008). There has been an exponential increase in the trend of research involved in exploring the efficacy of curcumin in animal models of major depression.

Aoi et al. (1986) reported a difference in the amount of curcumin found among individuals of *C. longa*. Ideally, the strains with a high content of curcumin should be selected, but it

Table 1. Sample list, accession numbers of the *atpF* intron in chloroplast DNA and curcumin content

No.	Species	Locality	*atpF* type	Curcumin content[1)] 2008	Curcumin content[1)] 2009
1	*Curcuma longa* (Kochi)	Japan, Kochi Pref.	I[2)]	357.6[b]	392.2[b]
2	*C. longa* (Tanegashima)	Japan, Kagoshima Pref., Tanegashima Island	I	391.7[b]	361.3[b]
3	*C. longa* (Wakayama A)	Japan, Wakayama Pref.	I	388.3[b]	374.5[b]
4	*C. longa* (Wakayama B)	Japan, Wakayama Pref.	I	0.7[b]	1.3[b]
5	*C. longa* (Wakayama C)	Japan, Wakayama Pref.	I	395.5[b]	389.5[b]
6	*C. longa* (Okinawa A)	Japan, Okinawa Pref.	I	364.0[b]	347.1[b]
7	*C. longa* (Okinawa B)	Japan, Okinawa Pref.	I	373.1[b]	305.0[b]
8	*C. longa* (Indonesia B)	Indonesia, West Java, Bogor	I	336.6[b]	309.5[b]
9	*C. longa* (Indonesia A)	Indonesia, West Java, Bogor	II	2677.8[a]	3059.1[a]
10	*C. longa* (Indonesia C)	Indonesia, West Java, Bogor	II	—[3)]	2315.1[a]
11	*C. longa* (Vietnam A)	Vietnam	II	—	2976.7[a]
12	*C. longa* (Vietnam B)	Vietnam	II	—	3198.4[a]

[1)] Curcumin content in primary branch rhizomes (mg/100g).

[2)] Type of the *atpF* intron in chloroplast DNA. I: accession number AB551928. II: accession number AB551927.

[3)] Not cultivated.

Values followed by the same latter in a column are not signigicantly different at 5% level by nonparametric test.

is difficult to distinguish strains in this species containing high amounts of curcumin because morphological characteristics of rhizomes are highly similar among them. Although there is a need for genetic markers to select strains with high curcumin content from any individuals of this species, reliable markers remain unclear. It is difficult to conduct sequencing analysis using many samples due to time and cost, therefore, it is necessary to develop the prompt method to identify strains within this species containing high amounts of curcumin.

Here, we investigated the insertion/deletion (indel) pattern of chloroplast DNA (cpDNA) by PCR amplification and the digestion pattern of cpDNA by PCR-RFLP analysis from individuals from various individuals of *C. longa* and evaluated the validity of cpDNA mutation for identification of strains with a high content of curcumin.

Materials and Methods

Cultivation experiments

The experiments were carried out in 2008 and 2009 at the Faculty of Agriculture, Kochi University, Japan using 12 *C. longa* (Table 1). Rhizomes were transplanted in late May for both years. For fertilizer dressing, a total of 1.5 kg/a of N, 0.6 kg/a of P_2O_5, and 1.4 kg/a of K_2O was applied over two years. In addition, 200 kg/a of compost fertilizer, 15 kg/a of magnesia lime, and 30 kg/a of chicken droppings were applied. The experimental plots were arranged in a randomized complete design with two replicates, which formed three rows. Due to a lack of seed rhizomes, some strains of *C. longa* were examined with/without replicates using two or three rows.

Samples were harvested in early December for two years. The curcumin content (mg/100g) of primary branch rhizomes was measured by using high-performance liquid chromatography (HPLC), according to the method described by Sato et al. (2004).

Molecular analysis

Total DNA was isolated from fresh root with a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, CA, USA), according to the manufacturer´s protocols. The PCR reaction mix contained approximately 100 to 200 ng of total DNA, 1 µM of each primer, 200 mM of

Table 2. Accession numbers of *matK* using phylogenetic analysis of *Curcuma* and outgroup

Species	*matK*	
	Accession No.	Reference
Curcuma aeruginosa	AF478840	Kress et al. (2002)
C. amarissima	AB047751	Cao et al. (Unpubl.)
C. aromatica	AB047731	Cao et al. (2001)
C. aromatica (Kochi)	AB551929	this study
C. aromatica (Tanegashima)	AB551929	this study
C. aromatica (Okinawa)	AB551929	this study
C. attenuata	GQ248110	Hollingsworth et al. (Unpubl.)
C. bicolor	AF478837	Kress et al. (2002)
C. chuanezhu	AB047736	Cao et al. (2001)
C. chuanhuangjiang	AB047732	Cao et al. (2001)
C. chuanyujin	AB047733	Cao et al. (2001)
C. comosa	AF478838	Kress et al. (2002)
C. elata	AB047747	Cao et al. (Unpubl.)
C. exigua	AB047750	Cao et al. (Unpubl.)
C. kwangsiensis	AB047745	Cao et al. (Unpubl.)
C. longa (Kochi)	AB551930	this study
C. longa (Tanegashima)	AB551930	this study
C. longa (Wakayama A)	AB551930	this study
C. longa (Wakayama B)	AB551931	this study
C. longa (Wakayama C)	AB551930	this study
C. longa (Okinawa A)	AB551930	this study
C. longa (Okinawa B)	AB551930	this study
C. longa (Indonesia A)	AB551930	this study
C. longa (Indonesia B)	AB551930	this study
C. longa (Indonesia C)	AB551930	this study
C. longa (Vietnam A)	AB551930	this study
C. longa (Vietnam B)	AB551930	this study
C. longa	AB047738	Cao et al. (2001)
C. phaeocaulis	AB047735	Cao et al. (2001)
C. roscoeana	AF478839	Kress et al. (2002)
C. sichuanensis	AB047740	Cao et al. (Unpubl.)
C. thorelii	AF478841	Kress et al. (2002)
C. wenyujin	AB047746	Cao et al. (2001)
C. xanthorrhiza	AB047752	Cao et al. (Unpubl.)
C. yunnanensis	AB047749	Cao et al. (Unpubl.)
C. zedoaria	AB551932	this study
C. zedoaria A	AB047734	Cao et al. (2001)
C. zedoaria B	AB047743	Cao et al. (2001)
Outgroup		
Boesenbergia rotunda	AF478827	Kress et al. (2002)
Cautleya spicata	AF478834	Kress et al. (2002)
Cornukaempferia aurantiflora	AF478835	Kress et al. (2002)
Curcumorpha longiflora	AF478842	Kress et al. (2002)
Kaempferia marginata	AB232054	Sitthithaworn and Komatsu (Unpubl.)
Scaphochlamys biloba	AF478889	Kress et al. (2002)
Zingiber mioga	AB047755	Cao et al. (Unpubl.)

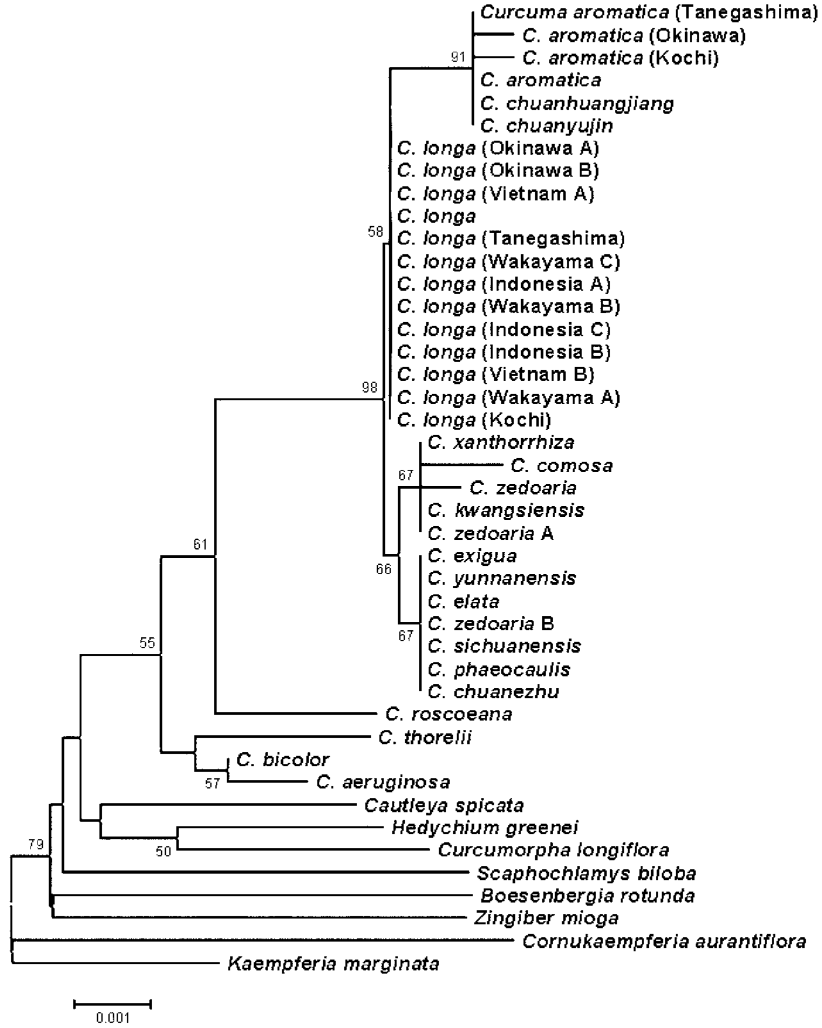
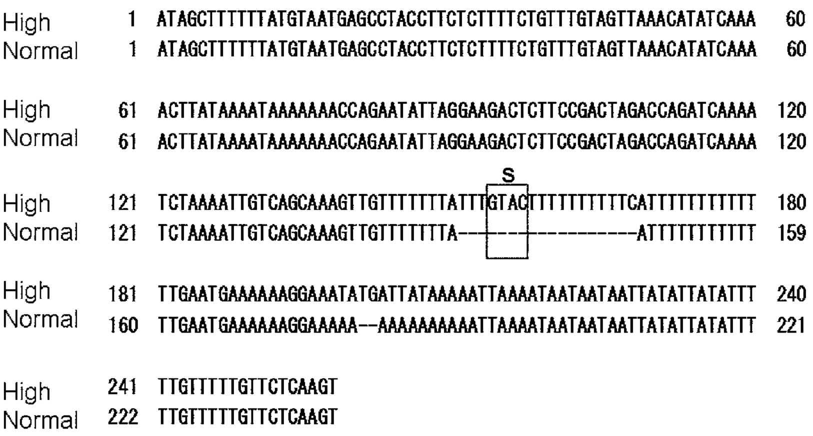


Fig. 1. Phylogenetic tree of *Curcuma* and its allied species using the neighbor-joining (NJ) method. The numbers below the branches indicate the bootstrap value. Sample names corresponded to Table 2.

each deoxynucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM of $MgCl_2$, and 1.25 units of Taq polymerase. Double-stranded DNA was amplified, after incubation at 94°C for 2 min, by 45 cycles of incubation at 94°C for 1 min, 48°C for 2 min and 72°C for 2 min, with final extension at 72°C for 15 min. We amplified the *matK* gene and the *atpF* intron in cpDNA with primers designed by Johnson and Soltis (1994) and Nishizawa and Watano (2000). After amplification, reaction mixtures were subjected to electrophoresis in 1% low melting-temperature agarose gels for purification of amplified products. We sequenced the purified PCR products using a Big Dye Terminator Cycle Sequencing Kit (ABI PRISM DNA Sequencing kit, Perkin Elmer Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100-Avant Genetic Analyzer according to the manufacturer′s instructions. For sequencing, we used the same primers as those used for amplification.



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                                                  S
High     1  ATAGCTTTTTTATGTAATGAGCCTACCTTCTCTTTTCTGTTTGTAGTTAAACATATCAAA   60
Normal   1  ATAGCTTTTTTATGTAATGAGCCTACCTTCTCTTTTCTGTTTGTAGTTAAACATATCAAA   60

High    61  ACTTATAAAATAAAAAAACCAGAATATTAGGAAGACTCTTCCGACTAGACCAGATCAAAA  120
Normal  61  ACTTATAAAATAAAAAAACCAGAATATTAGGAAGACTCTTCCGACTAGACCAGATCAAAA  120

High   121  TCTAAAATTGTCAGCAAAGTTGTTTTTTTATTTGTACTTTTTTTTTCATTTTTTTTTTT  180
Normal 121  TCTAAAATTGTCAGCAAAGTTGTTTTTTTA-----------------ATTTTTTTTTTT  159

High   181  TTGAATGAAAAAAGGAAATATGATTATAAAAATTAAAATAATAATAATTATATTATATTT  240
Normal 160  TTGAATGAAAAAAGGAAAAA--AAAAAAAAAATTAAAATAATAATAATTATATTATATTT  221

High   241  TTGTTTTTGTTCTCAAGT
Normal 222  TTGTTTTTGTTCTCAAGT
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Fig. 2. Expected restriction sites of *ScaI* for molecular characteristics of the *atpF* intron of cpDNA by PCR-RFLP. S. Restriction site.

To construct a phylogenetic tree based on *matK* sequences of *Curcuma* and its allied species (Table 2), the amplified regions (minus the length of the primers) were aligned using Clustal W (Thompson et al. 1994) and were improved manually using MEGA 4 (Tamura et al. 2007). Phylogenetic relationships were analysed using the neighbor-joining (NJ) method with paup* 4.08b (Swofford 2001). The NJ analyses were performed using MEGA 4 with Kimura′s two-parameter model. For the NJ analyses, bootstrapping with 1000 pseudo-replicates was chosen to examine the robustness of the clades and their phylogenetic relationships.

In the *atpF* intron in cpDNA, we carried out the PCR-RFLP analysis after checking the sequencing results and alignments. The result of alignments indicated that an autapomorphic cpDNA character of them was the presence of a restriction site of *ScaI*. After the designation of restriction sites, amplified products were digested by *ScaI* at 37 °C for more than an hour. Digested DNAs were separated on 1.5% agarose gel and the size of each band was determined.

Results and Discussion

Indonesia A and C and Vietnam A and B of *Curcuma longa* accumulated a high content of curcumin. However, *C. longa* (Wakayama B) showed little curcumin content (Table 1). This result confirms Aoi et al. (1986) in which there was a difference in the amount of curcumin among individuals of this species. Therefore, there was a need to find out nucleotide polymorphisms among these samples to identify lines with a high content of curcumin.

In general, phylogenetic analysis complements and often outperforms similarity searches, identifying variants and patterns in nucleotide sequence, when addressing sequence identity, especially the reference database of monocotyledonous plants does hold high matches in the *matK* gene of cpDNA (Tamura et al. 2004). Moreover, Selvaraj et al. (2008) claimed that the *matK* gene is a good candidate for DNA barcoding of *Zingiberaceae* including the genus *Curcuma*. Therefore, we determined sequences of the *matK* gene of *C. longa* and its allied species to identify this species. We used seven outgroups in our phylogenetic analysis; *Boesenbergia rotunda*, *Cautleya spicata*, *Cornukaempferia aurantiflora*, *Curcumorpha longiflora*, *Hedychium greenei*, *Scaphochlamys biloba* and *Zingiber mioga*. The length of the *matK* gene of all individuals including outgroups varied from 1831 bp (*C. longa*, Wakayama B) to 1846 bp (*C. thorelii*). The result of phylogenetic

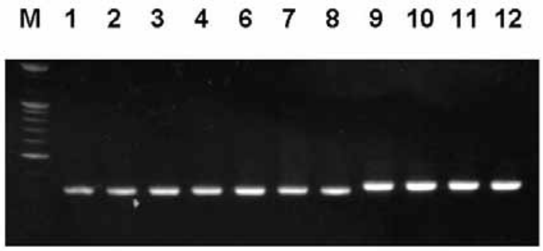

Fig. 3. The length variation of the *atpF* intron of cpDNA in *Curcuma longa*. M. Size marker. Numbers correspond to those of Table 1.

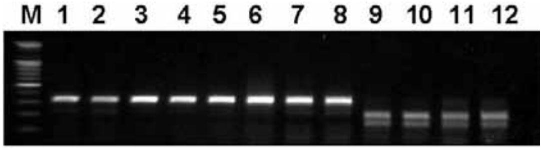

Fig. 4. PCR-RFLP profile of *Curcuma longa*. M. Size marker. Numbers correspond to those of Table 1.

analysis indicated that all *Curcuma longa* used in this study had the same of the *matK* gene (Fig. 1). Moreover, there were no same species in the genus with sequence as *C. longa*, indicating that this gene is available to identify whether a sample is *C. longa* or not. However, the *matK* gene was conserved within *C. longa* and could not be used to identify various strains of this species.

Recently, a short DNA have been used as moleculars marker for identifying the diversity that exists among plant and animal species (e.g., Hebert et al. 2003). In plants, many sequences of cpDNA have been utilized for studying sequence variation at infraspecific level and polymorphism of cpDNA has been used to study the DNA barcoding of various plant taxa (Nishizawa and Watano 2000). Therefore, we determined sequences of the *atpF* intron of *C. longa* based on Nishizawa and Watano (2000). The lengths of the *atpF* intron of all individuals of *C. longa* used in this study were 310 bp and 330 bp and there was a 20-bp indels in this sequence (Fig. 2). Our PCR amplification using various strains of *C. longa* indicates that this indel was specific to the strains with a high content of curcumin (Fig. 3). Moreover, although the first indel site of the plants with a high content of curcumin has one *ScaI* site, other low curcumin *C. longa* has no digesting site based on the sequence data (Fig. 2). Thus, we could anticipate the presence of the above digestion site for the samples used in this study. The result of digestion pattern indicated that all strains with a high content of curcumin and the other *C. longa* agreed with the expected patterns (Fig. 4). Since this indel appeared in all strains with a high content of curcumin of *C. longa*, the identification of the line with high curcumin content using the PCR procedure and the PCR-RFLP analysis may offer two means of identification the deletions and the digestion site in cpDNA. Moreover, these methods do not require sequencing analysis, therefore, these are prompt and inexpensive methods to identify the strain with a high content of curcumin within *C. longa*.

For exact identification of the strain with a high content of curcumin from various strains of *C. longa* in insufficient samples, the PCR procedure including these specific deletions in cpDNA and the PCR-RFLP analysis by *ScaI* are accurate and effective.

We wish to thank R. Arakawa, J. Yokoyama, T. Yoshida, A. Hirata, Y. Muramatsu, M. Saito, R. Ueda, K. Ohga, N. Yokoyama, M. Muroi and K. Matsuyama for providing much help. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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クルクミンは，主としてウコン *Curcuma longa* L. に含まれ，香辛料および薬用の材料として重要である．過去の報告では，ウコンのクルクミン量が種内系統間において異なることが示されている．種内の高クルクミン含有系統を識別することは形態学的に難しく，これらの分子同定法が必要である．そのため，高クルクミン含有系統を正確で簡易に識別するために，PCR 法によりウコンの葉緑体 DNA の塩基配列を決定した．その結果，高クルクミン含有系統のみ，*atpF* イントロン領域において合計 20 塩基の挿入または欠失が存在した．この変異部位には制限酵素である *ScaI* の認識部位が存在した．これらの結果より，*atpF* イントロンの PCR 増幅または *ScaI* による PCR-RFLP 法を用いることによって，ウコンの高クルクミン含有系統を正確に識別することが可能である．

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